Lipid and protein dynamics that shape nuclear envelope identity

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ABSTRACT **The nuclear envelope (NE) is continuous with the endoplasmic reticulum (ER), yet the NE carries out many functions distinct from those of bulk ER. This functional specialization depends on a unique protein composition that defines NE identity and must be both established and actively maintained. The NE undergoes extensive remodeling in interphase and mitosis, so mechanisms that seal NE holes and protect its unique composition are critical for maintaining its functions. New evidence shows that closure of NE holes relies on regulated de novo lipid synthesis, providing a link between lipid metabolism and generating and maintaining NE identity. Here, we review regulation of the lipid bilayers of the NE and suggest ways to generate lipid asymmetry across the NE despite its direct continuity with the ER. We also discuss the elusive mechanism of membrane fusion during nuclear pore complex (NPC) biogenesis. We propose a model in which NPC biogenesis is carefully controlled to ensure that a permeability barrier has been established before membrane fusion, thereby avoiding a major threat to compartmentalization.**

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INTRODUCTION

The nuclear envelope (NE) is a shared and essential feature of the endomembrane systems in all eukaryotes. It has long been appreciated that the NE is a defining structure for cellular organization as it is responsible for the separation of nucleoplasm from cytoplasm (Figure 1A) and the respective biological processes including transcription and translation that are operative therein. However, the NE is anything but a simple barrier. Instead, the inner nuclear membrane (INM) is endowed with a specialized proteome that is dedicated to a plethora of critical cellular functions (Korfali *et al.*, 2012; Garapati and Mishra, 2018; Gerace and Tapia, 2018). These include the sensation and buffering of mechanical forces (Burke, 2018; Kirby and Lammerding, 2018), genome organization and regulation (Buchwalter *et al.*, 2019a), and lipid metabolism (Bahmanyar *et al.*, 2014; Bahmanyar, 2015; Barbosa *et al.*, 2015, 2019; Haider *et al.*, 2018; Romanauska and Kohler, 2018). From a toplogical perspective, the NE is a physical extension of the ER that encases chromatin via inner and outer nuclear membranes (INM/ONM) that have distinct protein compositions and are linked at sites of fusion where nuclear pore complexes (NPCs) reside (Ungricht and Kutay, 2017) (Figure 1A).

Given the diverse functionality of the NE, it is not surprising that a steadily growing and diverse list of human pathologies are caused by mutations in NE or INM-associated nuclear lamina proteins. These pathologies include movement disorders and myopathies (Dauer and Worman, 2009; Meinke and Schirmer, 2016), cases of severely reduced life span and progeria (Kubben and Misteli, 2017; Fichtman *et al.*, 2019), embryonic lethality (Turner and Schlieker, 2016), and lipodystrophies (Shackleton *et al.*, 2000). Disruption of NE stability is also common in cancer cells causing DNA damage, cancer-relevant chromosomal rearrangements, and the intiation of proinflammatory pathways (Lim *et al.*, 2016; Umbreit and Pellman, 2017; Hatch, 2018).

Studies tackling NE pathologies, together with investigations centered on NE proteins in model organisms or tissue culture

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Abbreviations used: CCTα, choline phosphate cytidylyltransferase; CHMP7, charged multivesicular body protein 7; CNEP-1, C-terminal nuclear envelope phosphatase-1; CTDNEP1, C-terminal domain nuclear envelope phosphatase 1; Elo2, fatty acid elongase; ESCRT, endosomal sorting complex required for transport; FG nup, phenylalanine-glycine-rich nucleoporin; INM, inner nuclear membrane; NE, nuclear envelope; NEP1R1, nuclear envelope phosphatase regulatory subunit 1; NPC, nuclear pore complex; ONM, outer nuclear membrane; PNS, perinuclear space; UPS, ubiquitin/proteasome system.

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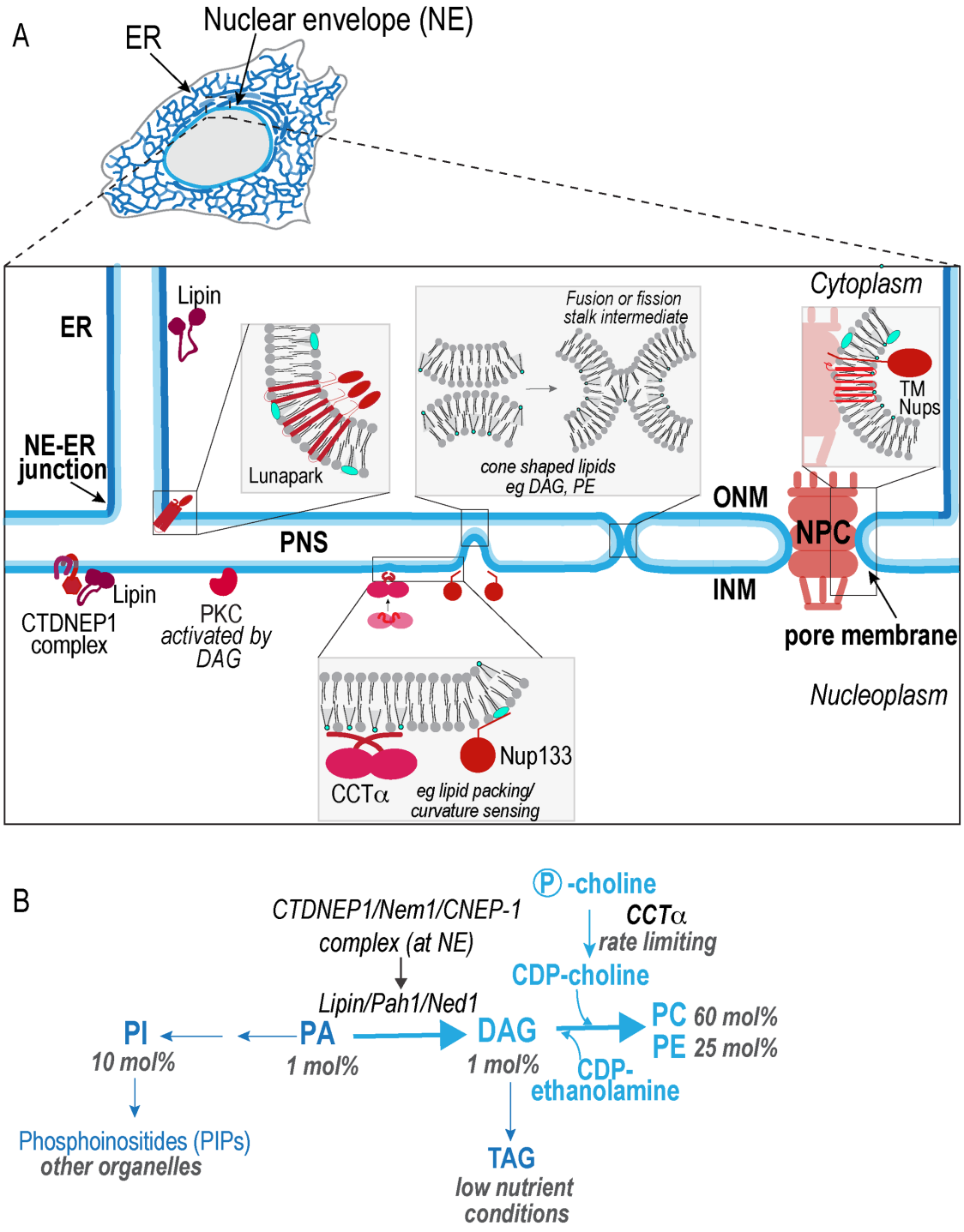

FIGURE 1: Features of the nuclear envelope and ER and regulation of and functions for lipid asymmetry at the inner nuclear membrane (INM). (A) Schematic of the continuous NE and ER membranes. The inner nuclear membrane (INM) facing the nucleoplasm and outer nuclear membrane (ONM) physically linked to the ER at NE-ER junctions are separated by a lumen designated perinuclear space (PNS). A nuclear pore complex (NPC) is located at a fusion point between the INM/ONM to generate the pore membrane. Highlighted in different shades of red are proteins that may regulate lipid trafficking between the NE and ER as well as enzymes and proteins that are regulated by or sense bilayer lipid composition (PKC and Nup133), or that regulate de novo lipid synthesis (CTDNEP1/lipin and CCTα). The curvature of the membrane bilayers may also play a role in restricting diffusion of lipid species past NE-ER junctions (negative curvature) or the pore membrane (positive curvature). Schematic of a membrane fusion reaction (middle) highlights membrane bending at each intermediate step. (B) The de novo glycerolipid synthesis pathway. Mol% for lipid species specific to ER/NE membranes is shown (van Meer *et al.*, 2008).

systems, revised the view of the NE to a dynamic membrane system that undergoes significant membrane remodeling even outside of open mitosis. This raises the question of what mechanisms are put to work to maintain or reestablish the NE permeabilty barrier, especially when NE integrity is perturbed. ESCRT (endosomal sorting complexes required for transport)-dependent processes are involved in the sealing of NE holes and have recently been discussed elsewhere (Campsteijn *et al.*, 2016; Webster and Lusk, 2016; Gatta and Carlton, 2019; Vietri *et al.*, 2020). We will discuss findings that connect lipid regulation and de novo lipid synthesis (see Figure 1B and more details below) to NE sealing (Kinugasa *et al.*, 2019; Lee *et al.*, 2020; Penfield *et al.*, 2020). Another emerging principle underlying NE dynamics pertains to the sculpting of its proteome by quality control mechanisms. These serve to degrade unwanted or misfolded proteins to maintain INM identity and safeguard protein homeostasis (Smoyer and Jaspersen, 2019). Being situated close to the nuclear transcriptional machinery, proteolytic mechanisms at the INM would also be ideally positioned to relay a perceived physiological demand to a transcriptional output, for example in the context of lipid homeostasis.

Finally, the best established facet of NE dynamics is the phenomenon of nuclear transport. NPCs traverse the NE and create a selective passageway through the INM and ONM and the enclosed perinuclear space (PNS) (Figure 1A). Regulated transport relies on nuclear transport receptors that enable cargo to passage through a meshwork of phenylalanine–glycine repeat nucleoporins (FG-nups) that establish a permeablity barrier between cytosolic and nuclear compartments. However, it is not known how NPC assembly is coordinated. In the following, we argue that the selective permeability barrier function relies on the fidelity and timing of membrane fusion between the INM and ONM during nuclear pore biogenesis, because an uncoordinated process could create a NE breach (Ungricht and Kutay, 2017).

In this Perspective, we discuss mechanisms critical for maintaining the identity and genome barrier function of the NE, with a focus on emerging roles of lipid metabolism and regulation of NPC biogenesis. Connected to these processes is the discovery of proteolytic systems that survey the NE proteome and may additionally play roles in the sharpening of compartmental identity and regulation of activities involved in lipid metabolism.

SPATIAL PARTITIONING OF BILAYER LIPIDS WITHIN THE CONTINUOUS NE AND ER

It is well known that the INM contains a unique set of integral membrane proteins; however, whether it also contains specific lipid species that contribute to its distinct identity and functions is not known. While the targeting of specific lipid kinases and phosphatases spatially restricts unique lipids within membranes of noncontiguous organelles (Behnia and Munro, 2005), the free diffusion of lipids within the continuous membranes of the ER and NE has been presumed to prevent their spatial segregation (Berg *et al.*, 2002). However, specific lipid species at the INM have been shown to support viral proliferation (Marschall *et al.*, 2011), NE dynamics (Hatch and Hetzer, 2014), de novo lipid synthesis (Haider *et al.*, 2018; Romanauska and Kohler, 2018) and NPC biogenesis (Drin *et al.*, 2007). Thus, despite the direct continuity of the lipid bilayers of the NE and ER, mechanisms may exist to selectively enrich and regulate specific lipid species at the INM. Aspects of some of these processes as well as potential mechanisms that drive lipid asymmetry at the NE are discussed below.

Lipid metabolizing enzymes associated with the surface of ER membranes produce the bilayer lipids of the NE, yet whether the products of these reactions are differentially distributed within the NE and ER is not known (Baumann and Walz, 2001; Fagone and Jackowski, 2009). This is in part because of the difficulty inherent in purifying the NE to homogeneity. Mass spectrometry analysis shows that the ER and NE mainly contain unsaturated glycerophospholipids and very low levels of the precursors required for their de novo synthesis (Figure 1B) (van Meer *et al.*, 2008). In metazoans, the de novo synthesis pathway for glycerophospholipids begins with formation of phosphatidic acid (PA) by the addition of two activated fatty acids to glycerol-3-phosphate, which can be dephosphorylated to form diacylglycerol (DAG) (Figure 1B). Both PA and DAG are precursors for the synthesis of membrane glycerophospholipids, phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylinositol (PI). Depending on nutrient conditions, DAG can also be converted to storage lipids (triglycerides) (Figure 1B) (Carman and Han, 2006; van Meer *et al.*, 2008; Fagone and Jackowski, 2009). Cholesterol and the biosynthetic intermediates to sphingolipids, ceramides and sphingoid bases, are also synthesized on the surface of the ER but represent an extremely low percentage of steady state ER/NE lipids—these lipids physically interact to form microdomains that enrich in export vesicles that traffic to the Golgi apparatus for further modifications (van Meer *et al.*, 2008). Because the length and degree of desaturation of fatty acids determines the flexibility and order of lipid bilayers, sphingolipids, which are composed of taller saturated fatty acids that pack tightly with cholesterol, are much more abundant in the thicker, stiffer plasma membrane than in internal membranes (Bigay and Antonny, 2012). Thus, the lipid composition of the ER/NE does not reflect its capacity for the synthesis of a diversity of lipids species, which is also under tight regulation by feedback mechanisms that safeguard its lipid content (Breslow, 2013; Goldstein and Brown, 2015).

The loose packing of unsaturated glycerolipids that populate the ER/NE provides a deformable environment that is suitable for insertion of newly synthesized proteins into ER membranes (Bigay and Antonny, 2012). Several lines of evidence suggest that regulation of fatty acid composition in the ER/NE may also support the function of the NE as an impermeable barrier to the genome. Overexpression of Elo2 to produce very long chain fatty acids suppresses NE ruptures in fission yeast (Kinugasa *et al.*, 2019), and accumulation of long-chain sphingoid base precursors to ceramide suppress NE morphology defects induced by aneuploidy in budding yeast and human cells (Hwang *et al.*, 2019). In addition, a sphingolipid hydrolase (Smpd4) that releases ceramide localizes to NPCs, suggesting a potential local role for sphingolipids and their precursors at the NE (Cheng *et al.*, 2019). These taller saturated lipids interact with cholesterol and, although a link between cholesterol and NE stability has not been made, the INM protein LBR (lamin B receptor) catalyzes a reaction in the production of cholesterol, suggesting that sterol synthesis may be in part regulated at the INM (Tsai *et al.*, 2016). A recent study showed that an increase in the production of lipids with unsaturated acyl chains by overexpression of Δ9 fatty acid desaturase Ole1 in *Schizosaccharomyces japonicus* rescues NE sealing defects, yet, unlike long-chain fatty acids, desaturated lipids are predicted to *increase* membrane fluidity (Lee *et al.*, 2020). Clearly, fatty acid composition influences NE stability but because the degree of saturation of lipids in ER membranes could regulate lipid enzyme activity to control lipid content and abundance, more work is required to understand the direct role for lipid saturation in NE stability.

Several lines of evidence suggest that the signaling and structural lipid DAG has specific functions at the INM (Figure 1B). The DAG-activated protein kinase C (PKC) has been shown to phosphorylate lamins for local and global disassembly; many of these

functions are reviewed elsewhere (Hatch and Hetzer, 2014). Experiments in budding yeast using PA and DAG sensors appended to nuclear localization signals also support a spatial enrichment of DAG at the INM (Romanauska and Kohler, 2018). Because budding yeast do not contain lamin-encoding genes (Peter and Stick, 2015), there are likely evolutionarily conserved functions for DAG at the INM that are independent of PKC activation for lamina disassembly. Consistent with this, Romanauska and Kohler (2018) showed that this pool of DAG can be utilized for de novo lipid synthesis and formation of lipid droplets at the INM, particularly under metabolic conditions that favor synthesis of storage lipids.

From yeast to metazoans, the reduction of lipin (Pah1 in *Saccharomyces cerevisiae*, Ned1 in fission yeasts) activity, the key enzyme that generates DAG for the production of glycerophospholipids or storage lipids (Figure 1B), causes abnormal NE expansion (Tange *et al.*, 2002; Golden *et al.*, 2009; Gorjanacz and Mattaj, 2009; Peterson *et al.*, 2011; Bahmanyar *et al.*, 2014) (Figure 1B). Lipin1 is regulated by multisite phosphorylation by the nutrient-sensing kinase mTORC1 and by the cyclin dependent kinase Cdk1 (Grimsey *et al.*, 2008; Peterson *et al.*, 2011). The highly conserved protein phosphatase complex CTDNEP1/NEP1R1 (Nem1/Spo7 in yeast and CNEP-1/NEPR-1 in *Caenorhabditis elegans*) dephosphorylates lipin (Santos-Rosa *et al.*, 2005; Han *et al.*, 2012; Bahmanyar *et al.* 2014) and in *C. elegans* CNEP-1 is enriched at the NE, suggesting that it locally regulates the NE-associated pool of lipin to produce DAG (Bahmanyar *et al.*, 2014). In addition to its catalytic functions, lipin1 regulates the transcription of lipid synthesis genes in response to nutrient availability through an unknown mechanism (Santos-Rosa *et al.*, 2005; Peterson *et al.*, 2011).

Insight into a role for lipin in forming the NE independent of its function in transcription came from studies in transcriptionally quiescent *C. elegans* embryos (Golden *et al.*, 2009; Gorjanacz and Mattaj, 2009; Bahmanyar *et al.*, 2014; Penfield *et al.*, 2020). CNEP-1 activation of lipin at the NE biases flux in the de novo glycerolipid synthesis pathway toward DAG and PC/PE synthesis at the expense of PI synthesis (Figure 1B) (Bahmanyar *et al.*, 2014; Penfield *et al.*, 2020). Ectopic ER sheets formed in *cnep-1* mutant embryos invade NE holes and prevent NE sealing (Penfield *et al.*, 2020). Loss of the NE adaptor protein CHMP7 for the ESCRT-III membrane scission machinery, which assembles on the negatively curved surface of NE holes to execute membrane fission (Vietri *et al.*, 2020), did not cause NE sealing defects on its own, but exacerbated the invasion of ER membranes and the defects in sealing resulting from deletion of *cnep-1*. These results led to a model that regulation of lipid synthesis cooperates with the ESCRT machinery to mediate NE sealing by restricting and remodeling ER membranes that feed into NE holes (Penfield *et al.*, 2020).

Depletion of PI producing enzymes rescued sealing defects in embryos deleted for *cnep-1* and ESCRT components, emphasizing the importance of lipid regulation in membrane remodeling to form the NE (Penfield *et al.*, 2020). It is not known whether the production of PI itself or PI derivatives or overall changes in the biophysical properties of the NE/ER affect ER structure and NE sealing under these conditions. The bulky head group of PI may prevent membrane fusion or impact signaling functions of PI or PI derivatives, which have been shown to play a role in ESCRT-dependent NE sealing (Ventimiglia *et al.*, 2018). It also remains possible that depletion of PI enzymes restores flux toward DAG production to rescue sealing defects, suggesting a specific role for DAG or the ratio of PA to DAG in NE sealing (Figure 1B). In *S. japonicus,* overexpression of the essential enzyme Ole1 (SCD1 in humans), which introduces double bonds into fatty acids that are channeled into PA for glycerolipid synthesis (Figure 1B), rescues NE sealing defects caused by loss of CHMP7 (*cmp7* in *S. japonicus),* further indicating a role for the de novo glycerolipid synthesis pathway in NE closure (Lee *et al.*, 2020).

Another major enzyme in de novo lipid synthesis that is regulated at the INM is the rate-limiting enzyme in PC synthesis, CCTα (Figure 1A) (Cornell, 2016). Changes in the ratio of PC to PE at the INM causes lipid packing defects that relieve protein autoinhibiton of nuclear CCTα through absorption of its membrane-binding domain (Haider *et al.*, 2018). The membrane-binding domain of CCTα locally senses changes in PC/PE ratios to restore PC homeostasis globally, suggesting that PE and PC rapidly diffuse and equilibrate between the ER and INM (Figure 1B). In support of this idea, Haider *et al.* (2018) found that CCTα does not have to localize to the INM to respond to lipid packing stress caused by an increase in the PE:PC ratio. Its localization to the nucleus confines its activity to INM lipids and keeps it from acting at other organelles. The flat surface of the INM relative to curved ER tubules may provide an environment more sensitive to changes in PC and PE levels (Cornell and Antonny, 2018).

Lipid synthesis and packing at the INM could facilitate the insertion of NPCs in interphase (Figure 1B). The nucleoporin Nup133 contains a membrane-binding domain ALPS motif that recognizes lipid packing defects (Drin *et al.*, 2007). Membrane bending generated at the base of intermediate NPC structures (Figure 1B) (Otsuka *et al.*, 2016; Otsuka and Ellenberg, 2018) is predicted to cause large packing defects (Bigay and Antonny, 2012) that may be sensed and stabilized by the Nup133 ALPS motif to promote pore assembly (Doucet *et al.*, 2010). Whether a membrane bending protein or the local conversion of lipids induces lipid packing defects to initiate pore assembly is not known. Completion of pore assembly through accumulation of a fusogenic lipid such as DAG may induce negative curvature to assist in fusion between the INM and ONM (Figure 1B).

A unique lipid composition at the INM requires a way to spatially partition lipids within a continuous membrane system (Figure 1A). Two ideas discussed here are as follows: 1) a physical barrier that reduces the timescale of lateral diffusion for specific lipids from one area (peripheral ER/ONM) to the other (INM) and 2) spatial restriction of synthetic enzymes to generate a continuum of concentrations high in one area (e.g., peripheral ER) relative to the other (e.g., INM).

Our current knowledge of a gradient of phospholipid concentrations is based on the increased activation of the PA phosphatase lipin by the phosphatase CNEP-1/CTDNEP1 at the NE (Figure 1) (Kim *et al.*, 2007; Bahmanyar *et al.*, 2014). Because PA serves as a precursor to PI, the resultant lower PA phosphatase activity of lipin on ER tubules located toward the cell periphery may permit the local production of PI from PA. Continuously restricting PI production to peripheral ER tubules balanced with transport of PI to other organelles would support a gradient of high PI concentrations at the cell periphery and low PI concentrations at the INM. The spatial restriction of lipin activity prevents PI accumulation and formation of ectopic ER sheets that interfere with the formation of a sealed nuclear compartment (Penfield *et al.*, 2020), a requirement for generating and maintaining NE identity.

In contrast to a gradient, a physical barrier would generate a distinct segregation of lipids between the peripheral ER and the INM. Two possible locations for a physical barrier are the fusion points between the ONM and INM (the pore membrane) or between the ONM and ER tubules (ER-NE junctions) (Figure 1B). The high negative curvature at ER-NE junctions might be sufficient to prevent the passage of inverted cone shaped lipids that prefer positive curvature, such as phosphoinositides, and more favorable

to the diffusion of cone shaped lipids such as DAG and PE (Figure 1B) (McMahon and Boucrot, 2015). In contrast, the positive curvature of the nuclear pore membrane imposes the opposite constraints (Figure 1B). Because the curvature of the luminal leaflet is the opposite of that of the cytoplasmic leaflet, transbilayer movement or "flip-flop" could also impact the ability of lipids to bypass their curvature constraints (Tsuji *et al.*, 2019).

In addition to membrane curvature, proteins could impose a physical barrier to the lateral diffusion of lipids (Trimble and Grinstein, 2015). The NPC is a stable structure associated with several transmembrane containing nucleoporins that might sterically hinder the diffusion of lipids with large or charged head groups (Figure 1B) (Rothballer and Kutay, 2013). Proteins that sit at ER-NE junctions may similarly serve as a critical intersection for the passage of lipids into the NE. The ER protein lunapark prefers the negatively curved membranes of three-way junctions and regulates their abundance in the ER, and so would be predicted to similarly control the number of ER-NE junctions that feed lipids into the NE (Figure 1B) (Chen *et al.*, 2015; Wang *et al.*, 2016). In fission yeast, lunapark regulates the bulk flow of lipids into the NE to support a constant nuclear-to-cytoplasmic-volume ratio (Kume *et al.*, 2019). ER junctions that intersect the NE may be destabilized by an increase in surface tension at the NE—volume expansion may loosen the barrier to promote bulk membrane flow. Thus, the topology (tubule-sheet fusion) and position of these junctions at the interface of the NE and ER are poised to serve a key role in defining the identity of these compartments.

Future work will determine how barriers to the diffusion of specific lipid species might function in concert with spatial restriction of lipid biosynthetic enzymes to establish and maintain a unique lipid composition of the NE. How unique lipid species in turn control the local dynamics and specialized functions of the NE is a major and open area of research essential to our fundamental understanding of organelle biogenesis and function in normal and diseased cells.

PROTEIN TURNOVER AT THE INM: AN ENHANCER OF COMPARTMENTAL IDENTITY?

While membranes play a defining role for compartment identity, compartment-specific proteins carry out specific functions. This poses a key problem for the NE of eukaryotes with an open mitosis, as the repeated breakdown of the NE and nuclear lamina during every mitotic cycle leads to loss of compartment identity by mixing of integral proteins of the ER and INM (Ellenberg *et al.*, 1997). The detailed events underlying NE reformation were reviewed recently (Ungricht and Kutay, 2017). Here we review mechanisms that maintain the NE proteome in interphase after the NE has formed, which is critical for cellular homeostasis since INM proteins that became effectively diluted due to cell duplication or are constantly removed via proteolysis (the average half-life of INM proteins is ~3–4 d; Buchwalter *et al.*, 2019b) need to be replaced. Equally important, particular physiological situations may require tailored compositions of INM proteins, for example in the context of cellular differentiation or stress responses, requiring dynamic adjustments through synthesis, nuclear import, and degradation.

Once the NE has reformed and the permeability barrier of the NPC has been established, INM components replenish the INM protein pool. Those INM proteins that bind lamin can diffuse from the ER to the INM and the reformed nuclear lamina, where they are locally retained by specific interaction with lamins and chromatin (Boni *et al.*, 2015; Ungricht *et al.*, 2015). Importantly, the NPC imposes a diffusion barrier effectively separating the ER and contiguous ONM from the INM by restricting the passage to membrane proteins with cytosolic domains of less than 40–60 kDa (Ohba *et al.*, 2004; Ungricht *et al.*, 2015). The molecular basis accounting for this property of the NPC is currently unknown. Structural information on the NPC-membrane interface at higher resolution is needed to resolve this conundrum. While this diffusion barrier can effectively exclude sizable membrane proteins, membrane proteins with smaller cytosolic domains may also need to be excluded from the INM as their presence there could potentially interfere with INM function.

One possible mechanism to enhance INM identity is to selectively remove proteins that do not belong to the INM and have "breached" the first selectivity filter. Indeed, a specialized branch of the ubiquitin (Ub)/proteasome system (UPS) is operative at the INM in both lower (Deng and Hochstrasser, 2006; Foresti *et al.*, 2014; Khmelinskii *et al.*, 2014) and higher (Tsai *et al.*, 2016; Buchwalter *et al.*, 2019b) eukaryotes, where it performs functions analogous to the ER-associated degradation (ERAD) machinery (Smoyer and Jaspersen, 2019). In brief, a typically polytopic Ub E3 ligase recognizes the substrate destined for degradation and mediates its ubiquitylation in conjunction with an Ub-conjugating enzyme. The ubiquitylated substrate is then extracted from the membrane and delivered to the 26S proteasome for degradation (Vembar and Brodsky, 2008). In yeast, the Asi1/2/3 complex and Doa10 are the E3 ligases responsible for turnover of INM proteins (Deng and Hochstrasser, 2006; Foresti *et al.*, 2014; Khmelinskii *et al.*, 2014) while the AAA+ ATPase Cdc48 and its human orthologue p97 mobilize the polyubiquitylated substrate from the INM in yeast and human cells, respectively (Foresti *et al.*, 2014; Tsai *et al.*, 2016) (Figure 2). The E3 ligases responsible for INM turnover in mammalian cells remain to be identified, though recently developed methodology relying on LBR-based model substrates for INM turnover may aid in their identification through proteomics-based or genome-wide screening approaches (Tsai *et al.*, 2019). Intriguingly, proteasomes were directly observed in association with the NE and in juxtaposition to nuclear pores in *Chlamydomonas reinhardtii* via cryo-EM tomography (Albert *et al.*, 2017), suggesting important roles for proteolytic systems at these sites. In yeast, the Asi2 subunit specifically recognizes transmembrane domains of orphan subunits of mislocalized ER proteins, leading to their removal from the INM through ubiquitylation, extraction by Cdc48, and subsequent turnover by the 26S proteasome (Natarajan *et al.*, 2019). It will be interesting to test whether the concept of "proteolytic sharpening" of compartmental identity via removal of proteins that "leak" into INM territory extends to the NE of mammalian cells, or even to other compartments.

The turnover of polytopic membrane proteins from the INM of mammalian cells can be very rapid. The half-life of truncated LBR variants is about ~10–15 min (Tsai *et al.*, 2016), which is significantly faster than the turnover of unrelated polytopic ERAD substrates, typically in the range of hours (Cambridge *et al.*, 2011). Thus, the INM-resident proteolytic system would be perfectly suited for rapid regulatory switches to initiate, for example, transcriptional responses following physiological stimuli. Given that regulatory proteolysis, such as the processing of transcription factors, plays key roles in mounting transcriptional responses to regulate lipid abundance (Goldstein and Brown, 2015) and phospholipid saturation (Rape *et al.*, 2001), it is tempting to speculate that additional roles of the UPS in lipid sensing or regulation will be identified at the INM. The close proximity to the transcriptional machinery would make the NE an ideal relay station to sense and amplify changes in membrane composition, through either alterations in lipid composition or membrane fluidity caused by packing defects. Similar functions linking mechanosensation to a transcriptional output can be envisioned.

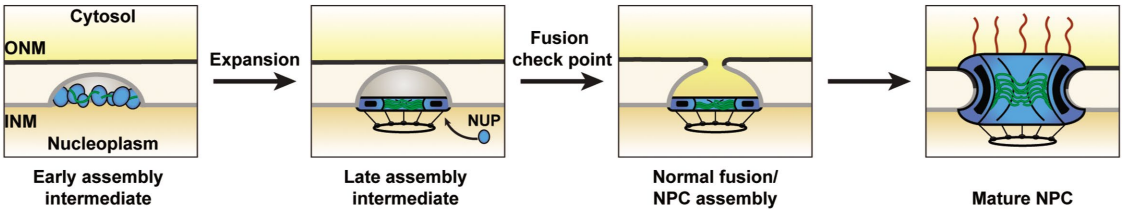

FIGURE 2: A fusion checkpoint for nuclear pore biogenesis. Nuclear pore assembly after nuclear envelope reformation proceeds through an inside-out protrusion mechanism starting from the nuclear side. Early assembly intermediates contain NPC constituents that deform the inner nuclear membrane (INM). This structure needs to expand to bring the INM and outer nuclear membrane (ONM) in close proximity for fusion. Either the integrity of the NPC assembly intermediate is sensed or the transport competence of a late assembly intermediate is required to initiate fusion. This hypothetical checkpoint mechanism would prevent a transient perturbation of NE integrity and can potentially explain why multiple, distinct NPC assembly defects lead to NE blebs resembling late assembly intermediates that got arrested before fusion (see the text).

HOW TO INSTALL NPCS WHILE PREVENTING A BREACH: A FUSION CHECKPOINT?

The integrity of the NE and its permeability barrier are under constant threat from mechanical stress, due to defects in lipid packing and through manipulation by pathogens that breach the NE during their life cycle. Over the course of evolution, eukaryotes have therefore evolved multiple mechanisms to prevent or counteract these threats. Mechanical reinforcements are key to maintaining NE integrity. These rely in part on the nuclear lamina, a meshwork of lamin-class intermedate filaments that are connected to the INM by association with INM proteins, as well as LINC (linker of the nucleoskeleton and cytoskeleton) complexes that bridge the NE and connect the nuclear lamina to cytosolic components of the cytoskeleton (Burke and Stewart, 2014; Hatch and Hetzer, 2016; de Leeuw *et al.*, 2018; Kirby and Lammerding, 2018; Stephens *et al.*, 2019). In addition, surveillance mechanisms exist in both yeast and mammalian cells that detect defects in NE integrity and recruit ESCRT machinery to reseal the NE membrane (Olmos *et al.*, 2015; Vietri *et al.*, 2015; Thaller *et al.*, 2019).

In higher eukaryotes, the occurrence of open mitosis by definition involves NE breakdown, representing a challenge for cellular compartmentalization. It is therefore not surprising that the reformation of the NE is an extremely rapid process that is completed within minutes after anaphase onset (Lu *et al.*, 2011; Otsuka *et al.*, 2018; Rampello *et al.*, 2020). Cell division poses yet another problem for NE integrity: daughter cells need to replenish the number of NPCs that had been diluted in the process, and these new channels must perforate both membranes of the NE. At least two mechanistically distinct processes account for NPC assembly in dividing mammalian cells, referred to as postmitotic and interphase assembly, respectively (Doucet *et al.*, 2010; Weberruss and Antonin, 2016; Otsuka *et al.*, 2018). Postmitotic assembly occurs during NE reformation while interphase assembly starts immediately after the NE has reformed (Dultz and Ellenberg, 2010; Rampello *et al.*, 2020). In mature NPCs resulting from either mechanism, a dense meshwork of FG repeats in the center of the NPC channel establishes a selective permeability barrier that allows the passage of only smaller proteins up to 30–60 KDa (Timney *et al.*, 2016). Larger cargo requires appropriate sorting signals that are recognized by transport receptors (karyopherins) that mediate transport through the NPC (Wente and Rout, 2010; Schmidt and Gorlich, 2016). But what happens before the FG-nup network is completed? Stalled or slowly maturing NPC assembly intermediates could present a massive problem were they to traverse both NE membranes before the establishment of the FG-nup barrier, thus effectively creating a hole within the NE. Similarly, assembly defects might create long-lived, defective intermediates with properties similar to those of pore-forming toxins, a problem that can be counteracted by membrane sealing of defective assembly intermediates (Wente and Blobel, 1993; Webster *et al.*, 2014). However, considering that a significant portion of newly formed NPCs are installed *after* the NE has reformed by interphase insertion in early G1 (Maul *et al.*, 1972; Dultz and Ellenberg, 2010; Rampello *et al.*, 2020), even unperturbed assembly could transiently create several hundreds of unscheduled, potentially toxic perforations of the NE in a mammalian cell. In what follows, we propose possible solutions to this problem that are informed by recent studies on NPC assembly.

Interphase NPC insertion relies on an inside-out budding mechanism in which NPC assembly is initiated at the INM and proceeds through a dome-shaped INM intermediate, with NPC precursors being situated at the base of the dome (Otsuka *et al.*, 2016; Otsuka and Ellenberg, 2018) (Figure 2). The bulged INM then fuses with the ONM, and cytoplasmic fibrils are added only after this fusion has occurred (Figure 2). However, interphase NPC assembly is a rapid process that is difficult to resolve temporally by cryofixation of cells and EM tomography. The recent finding that the deletion of Torsin ATPases leads to an arrest in interphase NPC biogenesis offers a unique opportunity to characterize "frozen intermediates" of NPC assembly (Laudermilch *et al.*, 2016; Rampello *et al.*, 2020). Live cell imaging using lattice light sheet microscopy in Torsin-deficient cells suggests that the early stages of assembly at the INM happen synchronously and rapidly immediately after the NE has reformed (Rampello *et al.*, 2020). This process is usually dynamic and would normally proceed to the step of INM/ONM fusion in an orderly manner (Otsuka *et al.*, 2016). We propose that INM/ONM fusion represents a critical quality control checkpoint. In our model (Figure 2), fusion occurs *only* if NPC assembly has matured to a point where the permeability barrier requiring the presence of central channel FG nups is established, thereby avoiding perturbations of the barrier function of the NE. While a plethora of mutations in NPC components leads to NE blebs (for a review, see Thaller and Lusk, 2018), it is possible that many of these aberrant structures result from failure to pass a quality control step at or before the point of membrane fusion.

Going forward, it will be interesting to test whether such a quality control checkpoint does indeed occur and to explore how the completion of an NPC assembly intermediate that is ready for fusion can be perceived on the molecular level. Perhaps the presence of a component that is added at a late stage is perceived and relayed to the currently unknown fusogenic machinery, or one of the components added late is itself endowed with fusogenic properties. Alternatively, it may be that only when the assembly process proceeds

properly that the ONM and INM come within a fusogenic distance (expansion phase, Figure 2).

As a variation of the latter model, one strategy would be to block the INM and ONM from achieving fusogenic proximity until the NPC precursor has acquired transport competence. (the two membranes are normally separated by up to 50 nm; Voeltz and Prinz, 2007). A specific transport event relying on the presence of FG-nups might expand the bleb or dome lumen and thereby bring the INM and ONM in close contact.The targeted delivery of a fusion activator or a fusogen from the nucleoplasm into the bleb lumen that depends on on the local presence of a dense FG-nup network might be another feasible strategy to build such a "fusion checkpoint."

Indeed, the role of specific FG-nups is not restricted to the building a permeability barrier but also entails a "Velcro" function that is required for NPC assembly (Onischenko *et al.*, 2017). Thus, the recruitment of factors implicated in fusion could similarly depend on the presence of specific FG-nups. Notably, fusogenic properties of RNAs have been described (Khvorova *et al.*, 1999; Janas and Yarus, 2003), so the search for the fusogen—the "holy grail" of NPC biogenesis—does not necessarily need to be restricted to proteinaceous candidates.

While a role for Torsins as ER and NE-luminal ATPases in the context of fusogenic machinery was previously proposed (Laudermilch and Schlieker, 2016; Weberruss and Antonin, 2016; Chase *et al.*, 2017), it may seem peculiar that unicellular eukaryotes do not have obvious Torsin orthologues and can still assemble NPCs. The question arises why interphase NPC biogenesis is stalled prior to membrane fusion in Torsin-deficient cells. Perhaps nature found alternative solutions for INM/ONM fusion, as for example in the case of endocytosis. In higher eukaryotes, dynamin GTPases mediate fission during endocytosis, whereas lower eukaryotes lack dynamins and employ actin-based mechanisms for fission (Ferguson and De Camilli, 2012; Lacy *et al.*, 2018). In yeast, Brr6 and Brl1 locate to NPC assembly sites and their mutation elicits phenotypes consistent with a key role for INM/ONM fusion (Zhang *et al.*, 2018); however, no direct homologues have so far been reported in mammalian organisms.

Another, not mutually exclusive, possibility is that Torsins are required to relieve an inhibition of fusion, for example, as part of the checkpoint mechanism outlined above. In a wider biological context, it may make sense to control the INM/ONM step beyond its possible checkpoint function in assembly as a means to regulate specialized transport. Notably, NE blebs with electron densities of dimensions at their base that are similar to NPCs and assembly intermediates of Torsin-deficient cells have been observed in zygotes and early embryos (see Szollosi and Szollosi, 1988; Laudermilch *et al.*, 2016). Moreover, RNA-containing NE blebs were observed in NEs at the neuromuscular junction in *Drosophila melanogaster* (Speese *et al.*, 2012) and were functionally linked to Torsins (Jokhi *et al.*, 2013). Perhaps these structures do represent NPC assembly intermediates, but they may also serve to concentrate specialized nuclear export cargo that is stored in their lumen. These assemblies would therefore sit poised for fusion, which would occur rapidly and synchronously following appropriate stimuli. This type of mechanism could serve to sharpen temporal control of transcriptional programs in a manner reminiscent of synaptic signaling. As mentioned above, the lipid composition of the NE plays an important role in the process of INM/ONM fusion, and specialized lipids may therefore play additional roles in this fusion process. Notably, Torsins were recently linked to lipid metabolism (Grillet *et al.*, 2016; Shin *et al.*, 2019) and a TorsinA variant can deform unilamellar vesicles in vitro (Demircioglu *et al.*, 2019). Lipid-specific probes suitable for in situ analysis would be particularly well-suited to shed light on these dynamic processes, as these could circumvent complications arising from biochemical fractionations of NE membranes and the inability to discern INM and ONM in NE isolates.

In conclusion, multiple mechanisms cooperate to shape the identity of the NE as a distinct subcompartment of the ER, either through limiting diffusion of proteins and lipids between those subcompartments or through localized remodeling and removal of undesired constituents. At the same time, the dynamic nature of the NE—for example during NPC biogenesis—requires fast-acting surveillance mechanisms and possibly checkpoints to preserve the separation of the cytoplasm and nucleoplasm that underpins eukaryotic organization. Among the major gaps in our knowledge of NE architecture is the absence of detailed information—both structural and compositional—of the ER/NE junctions and the NPC/pore membrane interface. An interdisciplinary effort is required to identify proteins that stabilize the membrane curvature at these sites and to identify mechanisms that restrict the diffusion of membrane proteins and lipids between the ER, ONM, and INM. Through these and other endeavors we will learn how separate identities are established to enable functional specification and diversification. Ultimately, a better understanding of these processes will allow us to rationalize how perturbations of this dynamic organization give rise to human pathologies, possibly informing strategies for therapeutic intervention.

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